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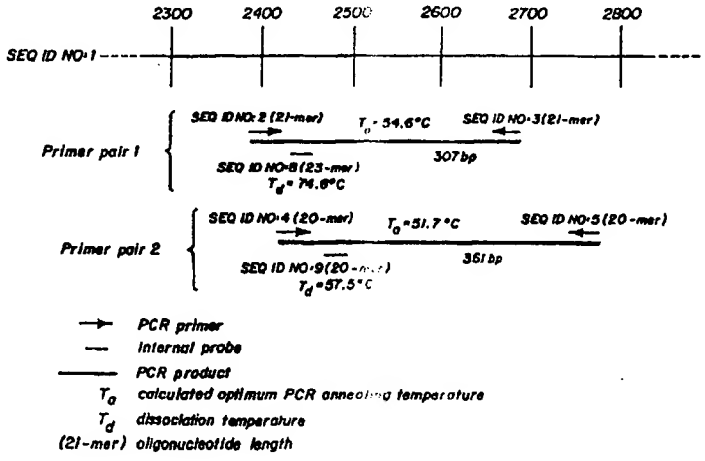

(57) Abstract

The subject invention provides processes and kits for detecting encysted forms of protozoa, particularly *Cryptosporidium* and *Giardia*, that are viable and infectious. To determine viability, cysts or oocysts are heated to a temperature that induces transcription of heat shock protein (HSP) genes. Alternatively, to determine infectivity the encysted forms are inoculated onto susceptible cell cultures. The viability or infectivity of the encysted forms can be determined by synthesizing a cDNA from an induced HSP RNA template using a primer that is specific for particular genus or species of protozoa, followed by enzymatic amplification of the cDNA. Alternatively, infectivity can be determined by amplifying HSP DNA from infected cells using a primer pair that is specific for a particular genus or species of protozoa. Amplified HSP DNA can be detected using probes that are specific for a protozoan species of interest, such as the human pathogens *C. parvum* and *G. lamblia*. Moreover, both *Cryptosporidium* and *Giardia* can be detected simultaneously by using two primer pairs in a multiplex amplification reaction.

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CRYPTOSPORIDIUM DETECTION METHOD

BACKGROUND

Protozoan parasites are a major cause of gastrointestinal disease. Within the last decade, the protozoa *Cryptosporidium* and *Giardia* have been increasingly associated with waterborne outbreaks of acute diarrhea. *Cryptosporidium parvum* is of particular concern because no known treatment of the illness is available at present. Moreover, in the immunocompromised host a *C. parvum* infection can lead to prolonged severe diarrhea, malnutrition, wasting, and death.

Cryptosporidium is an enteric coccidia, which has a multi-staged life cycle one to eight days in duration. The oocyst contains four sporozoites which, during normal infection, are released in the presence of bile salts and proteases. The sporozoites attach and penetrate intestinal epithelial cells. Once inside they develop into a rounded trophozoite in the area between the cytoplasmic membrane and the cytoplasm. Through asexual reproduction, the trophozoite (a type I meront) forms up to eight merozoites. The merozoites may then develop into a type II meront, which by asexual reproduction forms four merozoites. The second generation merozoites may develop into male (microgamont) or female (macrogamont) forms. The male form may lead to the sexual phase of the *Cryptosporidium* life cycle which culminates, *in vivo*, in the production of the environmentally resistant oocysts. These hardy structures possess a thick, double-layered protective cell wall which is resistant to most disinfectants, chlorine concentrations generally present in municipal water supplies, and temperatures between -4°C and 60°C.

Cryptosporidium is prevalent in most vertebrate groups. Domestic animals, such as rodents, kittens, puppies, and calves may constitute an important reservoir of the human *Cryptosporidium*. However, disease outbreaks in day-care centers, hospitals and urban family groups indicate that most human infections are transmitted person-to-person rather than via a zoonotic route. Since oocysts are found almost exclusively in stool, the transmission is undoubtedly fecal-oral. Moreover, the recovery of oocysts from both surface and drinking water suggests that indirect transmission via water is not uncommon.

Quantitative studies on the infectious dose for humans are currently limited. One study found that, in healthy volunteers, the infectious dose (ID_{50}) is 132 oocysts, with as few as 30 oocysts causing infection in 20% of individuals tested (DuPont et al., 1995).

However, the ID_{50} could be lower, e.g. one to ten oocysts, in more susceptible individuals.

Indeed, *Cryptosporidium* has been documented as a major cause of waterborne illness on numerous occasions. The largest outbreak occurred during the spring of 1993, in Milwaukee, Wisconsin, resulting in approximately 400,000 illnesses and 100 deaths (MacKenzie et al., 1994).

Cryptosporidium has been found to be somewhat ubiquitous in source waters. Two large multi-state surveys found *Cryptosporidium* in 50% of source waters tested (LeChevalier et al, 1991, and Rose et al., 1991). The Metropolitan Water District of Southern California (MWD) found *Cryptosporidium* in 24% of source water samples tested (unpublished data, 1992). *Cryptosporidium* was also found in 27%, 17%, and 6% of finished water samples in the LeChevalier, Rose, and MWD surveys, respectively.

These studies, surveys, and documented outbreaks clearly indicate that infectious *Cryptosporidium* may be found in source water and the efficiency of conventional water treatment needs to be closely monitored. Indeed, the occurrence of the causative agents *Cryptosporidium parvum* and *Giardia lamblia* in water supplies has become a critical issue for the water industry.

The current techniques for isolating *Cryptosporidium* and *Giardia* from water involve filtration and centrifugation to concentrate and purify oocysts and cysts, respectively, followed by immunofluorescence microscopy. Objects with the correct shape, dimensions, and fluorescence are confirmed by observation of internal structures using differential interference contrast microscopy. The limitations of these procedures includes loss of oocysts or cysts during isolation, resulting in recovery efficiencies ranging from 70 to 80 percent to less than one percent for *Cryptosporidium*. Moreover, the immunofluorescent assay (IFA) method cannot distinguish viable and potentially infective from non-viable or non-infective oocysts and cysts. Additional limitations of IFA include nonspecific antibody binding and cross-reactive antibody binding among human and animal infective species of *Cryptosporidium* or *Giardia*.

For the foregoing reasons, there is a need for an alternative method of detecting *Cryptosporidium* and *Giardia* pathogens that is rapid, sensitive, and specific. Ideally the method can distinguish among human and animal infective *Cryptosporidium* and/or *Giardia* species. Moreover, the alternative method should be able to determine if *Cryptosporidium* oocysts and/or *Giardia* cysts are viable and infective.

SUMMARY

The present invention is directed to detection methods and kits that satisfy these needs. Detection of viable and infective protozoa, particularly encysted forms of *Cryptosporidium* and *Giardia*, is accomplished by the enzymatic amplification of a target gene sequence, which encodes an inducible heat shock protein (HSP). The method exploits the speed, sensitivity, and specificity associated with an amplification procedure, such as polymerase chain reaction (PCR). Pathogenic forms of protozoa present in low copy numbers can be identified and distinguished from morphologically similar but non-pathogenic protozoa.

A first version of the invention is a method that selectively detects viable protozoan oocysts from a test sample. Oocysts are recovered from the test sample and the temperature is elevated to induce the transcription of heat shock protein (HSP) RNA. The oocysts are then lysed to release the RNA and DNA is removed from the lysate. A single-stranded cDNA is synthesized that is complementary to the HSP RNA by combining the RNA, a DNA polymerase having reverse transcriptase activity, four different deoxynucleotide triphosphates, and a first primer, which is complementary to a target HSP RNA sequence. A double-stranded cDNA sequence is then synthesized by incubating the single-stranded cDNA from the preceding step with a DNA polymerase, four different deoxynucleotide triphosphates, and a second primer. The second primer is complementary to a portion of the single-stranded cDNA and can initiate synthesis of a second cDNA strand. The double-stranded cDNA is then amplified to form an amplified target DNA by one of the amplification procedures well known in the art, such as PCR. The presence of viable oocysts is then determined by detecting the amplified target DNA.

A second version of the invention is a method that selectively detects infective protozoan oocysts in a sample. Cell cultures, which are susceptible to infection, are inoculated with a sample suspected of harboring infective oocysts. The cell culture is incubated under conditions that permit the infective oocysts to infect the susceptible cells. Cultured cells are then treated to gain access to the nucleic acids within the cells. An HSP gene sequence is selected as a target for detection. The nucleic acids from the cells serve as templates for at least two rounds of DNA synthesis, where a first primer and then a second primer hybridize with a portion of a first strand and a second strand of the HSP gene sequence. The primers initiate synthesis of a double-stranded polynucleotide sequence which is subsequently amplified by an appropriate amplification procedure. The presence of infective oocysts is then

determined by detecting the amplified target DNA.

The second version of the invention may amplify either DNA or RNA from the infected cells. Moreover, a quantitative assay for infective oocysts can be performed by adjusting, if necessary, the amount of oocysts used to inoculate the cells to a level that permits the enumeration of infection foci. Formation of discrete infection foci can be facilitated by adding an overlay to the cell culture, which retards the migration of infective organisms.

The invention can be adapted to detect HSP sequences conserved among different members of *Cryptosporidium* genus. In addition, the invention can be adapted to only detect the human pathogen, *C. parvum*. A third alternative is to simultaneously detect *Cryptosporidium* and *Giardia*. The specificity of the method is determined by the choice of primer pairs that specifically recognize HSP sequences for the protozoa of interest. In addition, the identification of a protozoa of interest can be confirmed by using oligonucleotide probes, which can hybridize with the amplified HSP target DNA.

The invention also provides kits for use in amplifying and detecting viable or infective *Cryptosporidium* and/or *Giardia* organisms. The kits can contain suitable amounts of the primers, or a suitable amount of the probe, or suitable amounts of the primers and probe.

DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims and accompanying drawings where:

Fig. 1 is a diagram showing the relative map positions of primer pair SEQ ID NO: 2 and SEQ ID NO: 3, primer pair SEQ ID NO: 4 and SEQ ID NO: 5, probe SEQ ID NO: 8, and probe SEQ ID NO: 9 within the HSP70 gene sequence SEQ ID NO: 1;

Fig. 2 shows amplification of DNA from *C. parvum* (lanes 1-5) and *C. muris* (lanes 6-10) by PCR using primer pair SEQ ID NO: 2 and SEQ ID NO: 3, which amplifies *Cryptosporidium* heat shock protein gene (HSP70) to give a 307 bp product (lanes 1 and 6), primer pair SEQ ID NO: 4 and SEQ ID NO: 5, which amplifies the *C. parvum* HSP70 gene to give a 361 bp product (lanes 2 and 7), primer pair LAX469F and LAX869R, which amplifies an undefined genomic region of *Cryptosporidium* DNA to give a 451 bp product (lanes 3 and 8), primer pair AWA722F and AWA1325R, which amplifies a portion of a *Cryptosporidium* 18S rRNA gene to give 556 bp product (lanes 4 and 9), and primer pair AWA 995F and AWA1206R, which amplifies another portion of a *Cryptosporidium* 18S rRNA gene to give a

256 bp product (lanes 5 and 10); Lane 11 contained a molecular weight marker.

Fig. 3 shows amplification of DNA from *C. parvum* and *G. lamblia* using primer pair SEQ ID NO: 2 and SEQ ID NO: 3 to give a 307 bp *Cryptosporidium* specific product (lane 1), primer pair SEQ ID NO: 4 and SEQ ID NO: 5 to give a *Cryptosporidium* specific 361 bp (lane 2), primers SEQ ID NO: 2 and SEQ ID NO: 3 combined with SEQ ID NO: 6 and SEQ ID NO: 7 for a multiplex PCR (lane 3), primers SEQ ID NO: 4 and SEQ ID NO: 5 combined with SEQ ID NO: 6 and SEQ ID NO: 7, which target heat shock protein genes of *C. parvum* (361 bp product) and *G. lamblia* (163 bp product), respectively; negative controls (lanes 5 and 6); and digoxigenin-labeled molecular weight markers (lane 7);

Fig. 4 shows a Southern blot of the gel shown in Fig. 6 hybridized with an internal oligonucleotide probe specific for both of the *C. parvum* amplification products (307 bp and 361 bp), which was labeled with fluorescein and detected by a chemiluminescent reaction mediated by alkaline phosphatase conjugated anti-fluorescein antibody;

Fig. 5 shows amplification, with primers SEQ ID NO: 2 and SEQ ID NO: 3 (lanes 1-3) and primers SEQ ID NO: 4 and SEQ ID NO: 5 (lanes 4-6), of DNA fragments from the following *Cryptosporidium* species.: *C. parvum* (lanes 1 and 4); *C. muris* (lanes 2 and 5); *C. baileyi* (lanes 3 and 6); Lane 7 shows 50, 150, 300, 500, 750, 1000-bp molecular size standards;

Fig. 6 shows a Southern blot of the gel shown in Fig. 5 hybridized with the SEQ ID NO: 9 oligonucleotide probe, which was labeled with fluorescein and detected by chemiluminescence using an anti-fluorescein antibody conjugated to alkaline phosphatase; and

Fig. 7 shows hybridization of the SEQ ID NO: 9 probe with the *C. parvum* specific PCR products of primers SEQ ID NO: 4 and SEQ ID NO: 5 using DNA extracted from 5 individual growth chambers containing mammalian cell cultures infected with *C. parvum* (lanes 1-5), uninfected cultured cells (lane 6), and environmental water concentrates seeded with *C. parvum* (lanes 7 and 8).

DETAILED DESCRIPTION

I. OVERVIEW

The present invention provides a method for amplifying and detecting viable and/or infective *Cryptosporidium* oocysts by monitoring the presence or absence of heat shock protein (HSP) genes and their transcripts. The heat shock response has a fundamental role during host invasion by parasites. When parasitic microorganisms infect another organism they

experience an increase in environmental temperature, because the body temperature of the host organism is higher than that of the surrounding environment. The physiological response of cells or entire organisms to this increased temperature is called the heat shock response and is characterized by increased transcription of the HSP genes (Maresca and Carratu, 1992). Increased expression (determined by elevated concentrations of mRNA) of HSP genes has been detected in a range of organisms including *Plasmodium*, *Trypanosoma*, *Candida*, and *Giardia* (Maresca and Carratu, 1992).

Since HSP gene transcription is a physiological response of living cells to an environmental stimulus, only viable *Cryptosporidia* are identified by amplifying and detecting HSP RNA transcripts from intact oocysts. Alternatively, infective *Cryptosporidia* are determined by first inoculating susceptible cell cultures with oocysts, and subsequently amplifying and detecting HSP DNA or RNA molecules from infected cells. A third alternative provides a method for simultaneously detecting *Cryptosporidium* and *Giardia* HSP gene amplification products.

II. RECOVERY OF OOCYSTS

The diagnosis of *Cryptosporidium* and *Giardia* is generally established by the recovery of *Cryptosporidium* oocysts and *Giardia* cysts from stool specimens. Alternatively, evidence for indirect transmission via contaminated water is provided by concentrating *Cryptosporidium* oocysts and *Giardia* cysts from water samples.

Cryptosporidium oocysts and *Giardia* cysts can be concentrated from water by a variety of methods. For example, a predetermined volume of water, e.g. 100 liters, can be filtered through a 1 μm nominal porosity yarn-wound polypropylene filter or its equivalent. The filtration flow rate is restricted to about 4 liters/min. Sampled filters are typically shipped on ice to analytical laboratories for analysis within 24 hours. Retained protozoa are eluted from the filter within 96 hours of collection with a buffered detergent solution, filter fibers are cut, teased and washed by hand or with the aid of a stomacher. Oocysts or cysts recovered in the eluent are concentrated by centrifugation and partially purified by flotation on a Percoll-sucrose solution with a specific gravity of 1.1. A portion of the purified material is placed on a membrane filter, tagged with antibody using the indirect staining method, and examined under UV microscopy. Specific criteria are used to identify cysts and oocysts including, immunofluorescence, size, shape, and internal morphology.

III. INFECTIVITY ASSAY

To determine infectivity, oocysts are inoculated onto susceptible cells, incubated under conditions that permit infection of cells to occur, and tested for the presence of HSP target DNA or RNA by an amplification procedure. Prior to the inoculation step the oocysts are decontaminated and, optionally, subjected to an excystation protocol.

A. Oocyst Pre-treatment

Oocysts recovered from water samples and stool specimens should be decontaminated to kill other microorganisms that may be present in the sample. Oocysts can be decontaminated by treatment with 10% chlorine bleach followed by washing with sterile 0.1% sodium thiosulfate to remove residual chlorine. If necessary, oocysts can also be treated with antibiotics prior to inoculation.

An optional excystation procedure may be included in oocyst pre-treatments to release sporozoites and improve infectivity. For example, oocysts are pelleted for 2 min at 5,000 x g in a microfuge, resuspended with ice-cold 10% chlorine bleach solution, and allowed to stand for 10 min on ice. The oocysts are then washed twice by successive pelleting and resuspension in sterile ice-cold saline solution. Finally, the decontaminated oocysts are resuspended in 1 ml of cell culture growth media.

An alternative excystation procedure includes the following steps. Purified oocysts are suspended in PBS and placed on ice. An equal volume of cold 40% chlorox bleach solution is added to the oocyst suspension and the mixture is allowed to stand on ice for 1 min. Oocysts are washed 2-3 times in cold phosphate buffered saline (PBS) to remove the bleach and pre-incubated in PBS for 1 hr at 37°C. An equal volume of prewarmed excystation fluid, consisting of 0.25% trypsin and 0.75% taurocholic acid, is added to the oocysts. The oocysts are incubated for up to 2 hrs at 37°C on a shaker.

When excystation is complete, excysted sporozoites are recovered by filtration through a syringe filter with a pore size of about 0.2 μm. Sporozoites are washed in Hanks Balanced Salt Solution (HBSS) to remove the excystation fluid.

B. Inoculation

Cryptosporidium oocysts or sporozoites are inoculated onto susceptible cells to determine whether the oocysts or sporozoites are capable of initiating an infection. Cells that are susceptible to *Cryptosporidium* infection include CaCo-2, HCT-8, and MDBK cells (ATCC Numbers HTB-37, CCL 244, and CCL-22, respectively). See also, Upton et al., 1994; Favennec et al., 1990. Susceptible cells can be grown as monolayers. When the

monolayers are 90% confluent, an inoculum is placed on the cells in a volume that is sufficient to cover the monolayer. The cells are then incubated at 37°C for 2 hours in 5% CO_2, 95% air. Inoculated monolayers are washed once with a saline-antibiotics solution to remove residual toxicity and re-incubated at 37°C.

Inoculation of *Giardia* cysts can be conducted similarly to *Cryptosporidium* infection. Excystation and decontaminating conditions are optimized for *Giardia* when Roswell Park Memorial Institute (RPMI) media supplemented with L-cysteine is used to stabilize cyst and trophozoite viability. The supernatant as well as the cell monolayer can be tested to determine if adequate numbers of *Giardia* trophozoites have attached to cell monolayers.

C. Quantitative Infectivity Assay

A quantitative infectivity assay can be performed by preparing cell monolayers attached to microscope slides and inoculating the cell with a measured dose of inoculum. Preferably, the slides are pre-treated with silane, collagen, BSA, laminin, fibronectin, or other cell attachment factors to increase cell adherence. Oocyst preparations are serially diluted until the inoculum contains less than about 1 oocyst per cell, i.e, a multiplicity of infection less than one (MOI $\langle$ 1).

Quantitative accuracy may be affected if mobile sporozoites and stage I or II merozoites are able to produce secondary infection sites. Short incubation periods, e.g., 24 hrs, may prevent some mobility of organisms. However, the movement of parasites is preferably restricted by the use of overlays. For example, soft agarose, agar, and methylcellulose overlays can be used to restrict movement of parasites only to adjacent cells (cell-to-cell transmission). As a result, discrete infection foci are produced, which can be enumerated.

D. Fixation Treatments

Subsequent cDNA synthesis, amplification, and detection procedures can occur *in situ*, i.e. within the confines of infected cells. Accordingly, the cells are fixed, in a manner that does not destroy cell morphology. An optimum fixation procedure will permit the reagents for subsequent reactions to diffuse into the semi-permeabilized cell. In addition, the reaction products should not be able to diffuse out of the semi-permeabilized cells.

A preferred fixation method includes treating the slides with methanol:acetic acid (3:1) at room temperature for 5 min. Following acidic methanol fixation, cells are

rehydrated in graded ethanol (95%, 70%, and 50%, 2 min each) and treated with 200 μl proteinase K (5 μg/ml) for 15 min at 37°C in a humid chamber. After the proteinase treatment, the slides are rinsed in PBS, pH 7.4 for 5 min. at room temperature. If the cells are to be used for detecting RNA, then 200 μl of an RNase-free DNase solution (about 750 U/ml) is layered on the cells, covered with a cover slip, and incubated in a humid chamber for about two to about four hours at room temperature. Alternatively, if only DNA is to be detected, the cells are treated in a similar manner with DNase-free RNase A. Following nuclease treatment, the cultured cells are washed with PBS and dehydrated in graded ethanol (50%, 70%, 95%, and 100%, 2 min each)

IV. PREPARATION OF NUCLEIC ACID EXTRACTS

Subsequent cDNA synthesis and amplification procedures can also be performed using nucleic acid containing extracts from cysts, oocysts, and infected cell cultures. Nucleic acids can be liberated from cysts, oocysts, and infected cell cultures by any method capable of lysing the cells. For example, the oocysts can be frozen in liquid nitrogen for 2 min., followed by thawing at 95°C for 5 min. The freeze/thaw cycle can be repeated, if necessary, and the lysate can be used directly in an amplification reaction.

If the extracts are to be used for detecting DNA, RNA can be removed from the lysate by treatment with DNase-free RNase A. Further purification of DNA from oocysts and infected cell cultures can be accomplished by additional extraction steps. For example, cells can be lysed in 50 mM Tris-HCl, 20 mM EDTA, pH 8, containing 2 mg/ml proteinase K and 0.5% sarkosyl, and incubated at 37°C for 1 h. Then, 5 M NaCl is added to give a final concentration of 1 M, and CTAB (hexadecyltrimethyl ammonium bromide) is added to a concentration of 1%. Following incubation at 65°C for 30 min, the lysate is subjected to at least one freeze/thaw cycle, and phenol/chloroform extraction. The DNA is precipitated by the addition of 0.6 vol, of isopropanol and the DNA precipitate is then washed with 70% ethanol.

If the extracts are to be used for detecting RNA, then DNA can be removed from the lysate by treatment with RNase-free DNase. Total RNA can be also be isolated from lysed cells by extraction with strong denaturants, such as guanidium thiocyanate, followed by centrifugation through a cesium chloride solution. Moreover, mRNA can be isolated using solid state particles attached to oligo-dT, which can select mRNA transcripts having a poly(A) tail.

V. SYNTHESIS OF cDNA

Expression of mRNA from HSP genes can be induced by incubating cysts, oocysts, or infected cells at about 37°C to about 42°C for 30 min. A cDNA, complementary to the HSP mRNA, can then be synthesized by a reverse transcription reaction. The basic components for synthesizing a first strand of cDNA includes an HSP RNA template, a DNA polymerase having reverse transcriptase activity, sufficient amounts of four different nucleotide triphosphates, e.g. dATP, dCTP, dGTP, dUTP, or their analogs, and a first primer. The target HSP RNA template can be extracted from lysed cysts, oocysts, or infected cells. Alternatively the HSP RNA can remain within fixed cells for an *in situ* reaction. Moreover, the first primer can hybridize with a portion of the HSP mRNA, thereby initiating the synthesis of the first cDNA.

The reverse transcriptase reactions typically contain: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2-5 mM $MgCl_2$, 1 mM each dATP, dCTP, dGTP, dUTP, 1 U/μl RNase inhibitor, 2.5 U/μl reverse transcriptase, 0.5 μM specific primer or 2.5 μM random primers, and total RNA or messenger RNA from at least one oocyst, in a 20 μl reaction. The reaction mixture can be incubated within a test tube or a multiwell plate. Alternatively, an *in situ* reaction is conducted by layering the mixture directly onto cells, placing a coverslip on top of the mixture, and sealing the edges of the coverslip with rubber cement or other suitable sealant. The reaction is preferably performed at 42°C for 15-60 min followed by 5 min at 99°C to stop the reaction.

VI. AMPLIFICATION

The amplification step of the present invention can be conducted using any of the amplification systems known in the art including the polymerase chain reaction system (U.S. Patent Nos. 4,683,195; 4,683,202; and 4,965,188), the ligase amplification system (PCT Patent Publication No. 89/09835) the self-sustained sequence replication system (U.S. Patent No. 5,409,818 and PCT Patent Publication No. 90/06995), the transcription-based amplification system (U.S. Patent No. 5,437,990), and the Qβ replicase system (U.S. Pat. No. 4,957,858). Each of the foregoing patents and publications is incorporated herein by reference.

A. Polymerase Chain Reaction

PCR is the preferred amplification system of the present invention. In the PCR amplification procedure a target HSP nucleic acid sequence is amplified by treating the double-

stranded polynucleotide with two oligonucleotide primers, each being complementary to one of the two strands. The primers hybridize with their complementary stands and extension products are synthesized using DNA polymerase and four different deoxynucleotide triphosphates. The DNA polymerase is preferably a thermostable enzyme, such as Taq, Tth, Pfu, or any other native, mutated, or deleted enzyme derived from a thermophilic organism. The extension products are separated from their complementary strands by denaturation at an elevated temperature, generally from about 80° to 100°C. The reaction mixture is repeatedly cycled between a low temperature annealing step, generally of from about 37° to 70°C, an intermediate temperature primer extension step, generally of from about 70° to 80°C, and a higher temperature denaturation step, generally of from about 80° to 100°C. If a thermostable DNA polymerase is used, the polymerase reaction can be cycled many times, typically 20-40 times, without needing additional enzyme.

B. In Situ Amplification

Reagent mixtures and conditions for *in situ* amplification are generally the same as those for standard PCR although concentrations of $MgCl_2$ and thermostable DNA polymerase are generally higher. Cycling parameters are determined by the optimal annealing temperature of the primers and the length of the PCR amplification product. Reaction sensitivity and specificity can be improved by using a "hot start", which prevents mis-priming and non-specific amplification. A hot start can be achieved by adding Taq polymerase only after the amplification reagents have reached 55°C. However, chemical hot start methods, e.g. by inclusion of dUTP and uracil-DNA glycosylase (UDG) or Taq antibodies (TaqStart™ Clontech), both of which inhibit PCR below 50°C, are preferred for *in situ* PCR due to their relative convenience.

For a typical *in situ* PCR, slides are heated to about 80°C and about 30 μl of preheated (80°C) amplification cocktail is layered onto the fixed cells. A coverslip is placed on top of the reaction mixture and the edges are sealed with rubber cement. A typical amplification cocktail contains: 10 mM Tris-HCl, pH 8.3; 50 mM KCl; about 2 mM to about 5 mM $MgCl_2$; about 250 μm each of dATP, dCTP, dGTP, and dUTP; about 3 μg/ml BSA; 10% glycerol; about 0.5 μM of each primer; and about 5 U to about 10 U of thermostable polymerase. Although other deoxynucleotide triphosphates may be included, dUTP is a preferred reaction component because UDG can be used to prevent carryover contamination of amplification reactions and as a chemical hot start. Temperature cycling can be performed in a

dedicated *in situ* PCR thermal cycler (PTC-100-16MS, MJ Research). The amplification cycle is repeated up to about 40 times with denaturation and extension taking place at about 94°C for about 1 min and 72°C for about 2 min, respectively. The annealing temperature will depend on the primers used.

VII. PRIMERS AND PROBES

Primers for the reverse transcription and amplification steps of the present invention are chosen to efficiently amplify nucleic acid sequences from organisms within a particular genus of protozoa, e.g. *Cryptosporidium* or *Giardia*. Alternatively, primers are chosen which only amplify a single protozoan species within a genus, e.g the human pathogens *C. parvum* or *G. lamblia*. The targets for amplification, i.e., the HSP sequences, are genes which can be easily induced to produce mRNA, since organisms capable of transcribing mRNA are viable and potentially infectious. If a *Cryptosporidium* oocyst or *Giardia* cyst is not viable then it will not produce HSP mRNA.

Primers and probes are preferably synthetic oligonucleotides, which can be prepared by an automated instrument (e.g, Applied Biosystems Inc., Foster City, CA). Alternatively, customized oligonucleotide primer and probes can be purchased from commercial suppliers, e.g., National Biosciences, Inc., Plymouth, MN.

Preferred primer pairs and probes target the HSP70 gene sequence of *C. parvum* (SEQ ID NO: 1, see also, Khramtsov et al., 1995). An alignment of HSP70 gene sequences from a range of organisms was done to find primers and probes for *Cryptosporidium*, which have an average sequence similarity of only 60% with mammalian HSP70 genes. Therefore, false positives due to non-specific amplification of host cell HSP70 genes are not a problem. Furthermore, DNA extracted from uninfected mammalian cells does not yield amplification products with these primers.

A most preferred primer pair (SEQ ID NO: 2 and SEQ ID NO: 3) can amplify DNA or RNA from many different *Cryptosporidium* species. Another most preferred primer pair (SEQ ID NO: 4 and SEQ ID NO: 5) is specific to *C. parvum*. Each set of primers has an internal oligonucleotide probe, which can be used to confirm the identity of the amplification product.

A. Cryptosporidium Primers

In one embodiment, primer pairs can amplify DNA or RNA from several *Cryptosporidium* species. A most preferred primer pair is:

SEQ ID NO: 2: CTG TTG CTT ATG GTG CTG CTG, and

SEQ ID NO: 3: CCT CTT GGT GCT GGT GGA ATA,

which typically gives a 307 base pair amplification product from *Cryptosporidium* nucleic acid extracts (see Fig. 1). The experimentally determined optimum annealing temperature of SEQ ID NO: 2 and SEQ ID NO: 3 is about 55°C.

In a second embodiment, the primer pairs are specific for *C. parvum*. The second embodiment is exemplified by the following most preferred primer pair:

SEQ ID NO: 4: AAA TGG TGA GCA ATC CTC TG, and

SEQ ID NO: 5: CTT GCT GCT CTT ACC AGT AC,

which typically gives a 361 base pair amplification product from *C. parvum* nucleic acid extracts (see Fig. 1). The experimentally determined optimum annealing temperature of SEQ ID NO: 4 and SEQ ID NO: 5 is about 55°C.

B. Multiplex Primers

In a third embodiment, primer pairs specific for *Giardia* are combined with *Cryptosporidium* specific pairs for a "multiplex" amplification. For example, the DNA sequence for a heat shock protein in *G. lamblia* that is unrelated to HSP 70, has been described (Aggarwal et al., 1990, incorporated herein by reference). Moreover, primers targeting the HSP gene have been described, which are suitable for the detection of viable *G. lamblia* cysts in water samples (Abbaszadegan et al., 1993, incorporated herein by reference) The following primers:

SEQ ID NO: 6: AGGGCTCCGGCATAACTTTCC, and

SEQ ID NO: 7: GTATCTGTGACCCGTCCGAG,

amplify a 163 base pair product from *G. lamblia*. The optimum annealing temperature for SEQ ID NO: 6 and SEQ ID NO: 7 is about 55°C and the optimum $MgCl_2$ concentration is 2.5 mM.

When *G. lamblia* specific primers, and *C. parvum* specific primers are combined in a single amplification reaction mixture, two amplification products are produced. For example, the primer pair SEQ ID NO: 6 and SEQ ID NO: 7 and primer pair SEQ ID NO: 4 and SEQ ID NO: 5 can be used to amplify two separate HSP sequences at an annealing temperature of about 52°C (see Fig. 3 lane 4). The first amplification product is a 361 base pair DNA fragment, corresponding to *C. parvum* HSP 70 sequences. The second

amplification product is a 163 base pair DNA fragment, corresponding to sequences of a *G. lamblia* heat shock protein gene that are unrelated to HSP 70.

VIII. DETECTION

The amplified HSP target DNA can be detected directly by any method that can distinguish among different lengths of DNA. Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide. Bands corresponding to the predicted length for amplified target DNA can then be detected by direct examination of the gel in ultraviolet light.

In addition, the DNA bands from an electrophoresed gel can be transferred to a membrane support by capillary action, followed by indirect detection using oligonucleotide probes. A typical transfer protocol includes denaturing the DNA within the gel using an alkaline solution, such as 0.4 M NaOH, 0.6 M NaCl, followed by a neutralization step in a buffer solution, e.g. 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5. The gel is then equilibrated with a high ionic strength transfer buffer, such as 10X SSC, wherein 1X SSC is 0.15 M NaCl, 0.015 M Na citrate. The denatured DNA can then be transferred from the gel to a membrane support by capillary blotting in transfer buffer.

A. Hybridization Probes

A preferred mode for detecting amplified target sequences is via hybridization to a single-stranded oligonucleotide probe which is sequence-complementary to a sequence located between the two selected oligonucleotide primers in the target HSP gene. The identity of the amplified extension products from each set of primers can thereby be confirmed using the sequence specific probes. Oligonucleotide probes are normally selected according to their ability to hybridize with an internal sequence of an amplified target DNA. Thus, probes that can detect the amplified products of the viability or infectivity assays are complementary to an amplified heat shock protein gene sequence, preferably HSP70.

A most preferred probe is an oligonucleotide, which specifically hybridizes with HSP70 sequences of *C. parvum*. The following two probes are exemplary of *C. parvum* specific probes:

SEQ ID NO: 8: AAA TGG TGA GCA ATC CTC TGC CG

SEQ ID NO: 9: CCA TTA TCA CTC GGT TTA GA

The first *C. parvum* HSP70 specific probe, SEQ ID NO: 8, contains sequences

corresponding to nucleotide base numbers 2423 to 2446 of SEQ ID NO: 1. The SEQ ID NO: 8 probe can be used to detect *C. parvum* specific sequences of any HSP70 targets having complementary sequences to nucleotide base numbers 2423 to 2446 of SEQ ID NO: 1. Consequently, SEQ ID NO: 8 can be used to detect the amplification products of the SEQ ID NO: 2 and SEQ ID NO: 3 primer pair (see Fig. 1).

The second *C. parvum* HSP70 specific probe, SEQ ID NO: 9, contains sequences corresponding to nucleotide base numbers 2475 to 2495 of SEQ ID NO: 1. The SEQ ID NO: 9 probe can be used to detected *C. parvum* specific sequences of any HSP70 targets having complementary sequences to nucleotide base numbers 2475 to 2495 of SEQ ID NO: 1. Accordingly, SEQ ID NO: 9 can be used to detect the amplification products of a variety of primer pairs, such as SEQ ID NO: 2 and SEQ ID NO: 3; SEQ ID NO: 2 and SEQ ID NO: 5; and SEQ ID NO: 4 and SEQ ID NO: 3 (see Fig. 1).

Similarly, when primers pairs, such as SEQ ID NO: 6 and SEQ ID NO: 7, are used to amplify *G. lamblia* specific sequences, an internal probe can be used to confirm the identity of the *Giardia* targets. For example, the following internal oligonucleotide probe can be used for detecting *G. lamblia* HSP targets.

SEQ ID NO: 10: CAGGCCTTGGCGTTCCCGAAG.

Giardia HSP probes are especially useful in a "multiplex" amplification procedure, which includes primers for both *Giardia* and *Cryptosporidium* target sequences. The *Giardia* HSP probes can then be used to distinguish *Giardia* specific amplification products from any other amplification products.

B. Hybridization of Southern Blots

Amplified target DNA that has been captured on a solid support, such as nylon or nitrocellulose membrane, can be detected by using a labeled hybridization probe. The probe can be labeled with a radioactive or fluorescent tag, or attached directly or indirectly to an enzyme molecule. Then, the membrane-bound amplified target DNA is incubated with the probe under hybridization conditions. Following hybridization, excess probe is washed away. If the hybridization probe is radioactively tagged, the remaining hybridized probe can be detected by autoradiography or scintillation counting. If the probe contains biotin or some other chemical group for which there are specific binding molecules, like avidin and antibodies, then the immobilized probe can be detected with an enzyme attached to the specific binding molecule, such as horseradish peroxidase or alkaline phosphatase attached to

streptavidin.

A preferred method of detection is via hybridization with a nonradioactive 5' digoxigenin (DIG)-labeled oligonucleotide probe. Following hybridization the solid support is washed with a high ionic strength buffer, such as 5X SSC, at about 70°C. The immobilized hybridization probe that remains after washing can be visualized by incubating the solid support with anti-DIG antibody conjugated to alkaline phosphatase, followed by addition of a chemiluminescent substrate, such as Lumigen-PPD (Boehringer Mannheim). The support is finally washed, sealed in Saran Wrap, and exposed to X-ray film to detect any chemiluminescence.

C. ***In Situ* Detection**

There are two approaches for the detection of *in situ* amplification products. The first *in situ* detection method is a direct technique, which involves incorporation of a label directly into the amplification product. For example, a reporter molecule such as digoxigenin [DIG]-dUTP or fluorescein-dUTP can be included in the amplification cocktail and incorporated into the amplification product. A simple immunochemical step using alkaline phosphatase- or peroxidase-conjugated anti-DIG then detects DIG labeled amplification products. Alternatively, fluorescein labeled amplification products can be detected by fluorescence microscopy or immunochemical methods.

The second *in situ* detection method is an indirect technique, which involves hybridization of a specific labeled probe to the amplification product after PCR. The label on the probe is then detected either by immunochemical methods or fluorescence microscopy. The indirect method is preferred because it has a higher specificity than direct *in situ* PCR. Moreover, by using multiple probes for *in situ* hybridization, each labeled with a different fluorescent molecule, e.g., fluorescein, rhodamine and couramin, each targeting different amplification products, the potential exists for detecting multiple target genes in a single sample.

By combining reverse transcription of RNA and *in situ* amplification with cell culture infectivity assays a method has been developed which allows the sensitive and specific detection of expressed *Cryptosporidium* and *Giardia* genes. Expression of the target genes indicates the presence of viable protozoa within cell cultures grown on microscope slides. In addition, such a method determines the infectivity potential of *Cryptosporidium* oocysts and *Giardia* cysts within about 48 to about 72 hours after inoculation.

IX. KITS

The primers and/or probes, used to amplify and detect viable or infective *Cryptosporidium* and/or *Giardia* organisms, can be conveniently packaged as kits. The kit may comprise suitable amounts of the primers, or a suitable amount of the probe, or suitable amounts of the primers and probe. In addition, kits can contain a suitable amount of at least one standard sample, which contains a known concentration of a *Cryptosporidium* or *Giardia* species, and a negative control sample substantially free of the protozoa of interest.

The methods and kits of the present invention have many advantages over previous methods, including the speed, sensitivity, and specificity associated with amplification procedures, such as PCR. Since the methods can detect only viable and infectious forms of *Cryptosporidium* and *Giardia*, the effectiveness of disinfection procedures can be monitored. Moreover, the human pathogen, *C. parvum*, can be distinguished from other *Cryptosporidia*, such as *C. muris* and *C. baileyi*, which only infect animal hosts.

EXAMPLES

Materials and Methods

Purified preparations of *C. parvum* oocysts and *G. lamblia* cysts were obtained from two commercial laboratories Parasitology Research Laboratories (PRL), Phoenix, Ariz. and Waterborne, Inc., New Orleans, LA. *C. muris* oocysts were generous gifts of J. Owens (United States Environmental Protection Agency, Cincinnati, Ohio) and are available commercially from PRL. *C. baileyi* oocysts were generous gifts of Dr. B. Blagburn (Auburn University, Auburn, Ala.) Cysts and oocysts were supplied as purified preparations stored in antibiotic solution or as unpurified concentrates and were stored at 4°C. Cyst and oocyst densities were determined by hemocytometer counting and lower densities were obtained by serial dilution of concentrated stocks.

Example 1

Specificity of Primer Pairs for *C. parvum* and *C. muris* Oocyst Dna

This example compares the specificity of primers directed to different portions of the HSP70 gene, an undefined genomic region of *Cryptosporidium* DNA, and the 18S rRNA gene, for *C.parvum* and *C.muris*.

DNA was extracted from *C. parvum* and *C. muris* oocysts by freezing in liquid nitrogen for 2 min, followed by thawing at 95°C for 5 min. Five μl of the freeze-thaw lysate (equivalent to about 1,000 cysts or oocysts) was added to individual amplification reactions,

which also contained: 10 m*M* Tris-HCl, pH 8.3; 50 m*M* KCl; 0.01% gelatin; 2.5 mM $MgCl_2$, 0.25 μM of each primer, 200 μM each of dATP, dCTP, dGTP and dUTP; and 2 U of Amplitaq® DNA polymerase (Perkin-Elmer, Foster City, CA) in a 100-μL volume.

The reactions were overlaid with two drops of sterile mineral oil (Sigma Chemical Co., St. Louis, MO). Hot start reactions were performed in a DNA Thermal Cycler model 480 (Perkin-Elmer) with denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 50°C and extension at 72°C for 1 min. A final extension incubation at 72°C for 5 min was included followed by 5 min at 5°C to stop the reactions.

PCR products (15% of the amplification reaction) were detected by standard agarose gel electrophoresis and ethidium bromide staining as shown in Figure 2. Primer pair SEQ ID NO: 2 and SEQ ID NO: 3, which is directed to a portion of the HSP70 gene, primed the amplification of a 307 base pair (bp) product from both *C. parvum* and *C. muris* DNA extracts (see Lanes 1 and 6 of Figure 2). In contrast, primer pair SEQ ID NO: 4 and SEQ ID NO: 5, which is directed to a different portion of HSP70 gene, primed the amplification of a 361 bp product from *C. parvum*, but not *C. muris* DNA extracts (see Lanes 2 and 7 of Figure 2).

These results compare favorably with primer pairs directed to the 18S rRNA gene (Awad-El-Kariem et al., 1994), which primed the synthesis of amplification products from *C. parvum* and C. *muris* extracts (see Fig. 2, lanes 4, 5, 9, and 10). In addition, primers directed to an unspecified genomic region of *Cryptosporidium* DNA (Laxer et al., 1991) primed a 451 base pair amplification product from *C. parvum* extracts (see Fig. 2, lane 3).

Thus, primer pair SEQ ID NO: 2 and SEQ ID NO: 3 exemplifies an HSP 70 primer pair that is specific for at least two different members of the *Cryptosporidium* genus, whereas the specificity of primer pair SEQ ID NO: 4 and SEQ ID NO: 5 was limited to *C. parvum*.

Example 2

Multiplex Primer Amplification of *C. parvum* and *G. lamblia* Followed by *C. parvum* Oligoprobe Detection

This example illustrates that a combination of two primer pairs directed to *C. parvum* and *G. lamblia* HSP sequences can accurately amplify both target sequences simultaneously. Moreover, the identity of the *C. parvum* amplification product can be

confirmed using a *C.parvum* specific oligonucleotide probe.

DNA was extracted from *C. parvum* oocysts and *G. lamblia* cysts by freezing in liquid nitrogen for 2 min, followed by thawing at 95°C for 5 min. The amplification reaction contained 10 m*M* Tris-HCl, pH 8.3; 50 m*M* KCl; 0.01% gelatin; 1.5 m*M* $MgCl_2$; 0.25 μM of each primer; 200 μM each of dATP, dCTP, dGTP and dUTP; and 2 U of Amplitaq® DNA polymerase (Perkin-Elmer, Foster City, CA) in a 100-μL volume with 5 μL template DNA. Negative control reactions contained sterile distilled water in place of template DNA..

The reactions were overlaid with two drops of sterile mineral oil (Sigma Chemical Co., St. Louis, MO). Hot start reactions were performed in a DNA Thermal Cycler model 480 (Perkin-Elmer) with denaturation at 94°C for 2 min. followed by 40 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 52°C, and extension at 72°C for 1 min. A final extension incubation at 72°C for 5 min was included, followed by 5 min at 5°C to stop the reactions.

PCR products (15% of the amplification reaction) were detected by standard agarose gel electrophoresis and ethidium bromide staining (see Fig. 3). Reactions primed only with SEQ ID NO: 2 and SEQ ID NO: 3 exhibited the expected 307 base pair product (Fig. 3, lane 1). Similarly, reactions primed only with SEQ ID NO: 4 and SEQ ID NO: 5 exhibited the expected 361 base pair product (Fig. 3, lane 2). Amplification products from reactions primed with both primer pair SEQ ID NO: 2 and SEQ ID NO: 3, and primer pair SEQ ID NO: 8 and SEQ ID NO: 9 were not visible on the ethidium bromide stained gel (see Fig. 3, lane 3). However, two amplification products, 361 base pairs and 163 base pairs in length, were detected from reactions primed with SEQ ID NO: 4 and SEQ ID NO: 5, and primer pair SEQ ID NO: 8 and SEQ ID NO: 9 (see Fig. 3, lane 4). The 361 base pair and 163 base pair bands correspond with the expected size of the amplification products for *C. parvum* and *G. lamblia*, respectively.

DNA was denatured by incubation of the gel for 30 min each in 0.4 M NaOH, 0.6 M NaCl followed by 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5 at room temperature. The gel was then incubated for 20 min in 10 x SSC (1X SSC is 0.15 M NaCl, 0.015 M Na citrate). Denatured DNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) by overnight capillary blotting in 10X SSC. Transferred DNA was cross-linked to the membrane by UV irradiation (120 mJ for 2 min) followed by drying at 80°C.

The membrane was prehybridized for 1 h in 20 ml of hybridization solution which

contained 5X SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% sarcosine, 0.02% SDS at 64°C. The membrane was then hybridized in fresh hybridization solution containing 50 pmoles of 5'-fluorescein labeled oligonucleotide probe SEQ ID NO: 9 for 18 h at 64°C in a rotary hybridization oven (Model 310, Robbins Scientific, Sunnyvale, CA). Stringency washes containing 20 m*M* Tris-HCl, pH 7.4, 0.01% SDS, and 5X SSC were performed at 70°C, twice for 15 min each.

Hybridized probe was detected with an anti-fluorescein alkaline phosphatase conjugate and a chemiluminescent substrate. Membranes were washed for 5 min in 20 ml of 0.3% Tween 20 followed by 30 min incubation in 100 ml of 1% blocking reagent. Both of these solutions were made up in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 and all incubations were at 23°C in a rotary hybridization oven. Fluorescein labeled anti-Digoxigenin (1.5 U, Boehringer Mannheim) was added in 20 ml of 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1% blocking reagent and incubated for 15 min to label the molecular size markers. Anti-fluorescein alkaline phosphatase (1.5 U, Boehringer Mannheim) was added to the membrane in 20 ml of 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1% blocking reagent and incubated for 30 min. The membranes were washed twice in 100 ml of 0.3% Tween 20 followed by 5 min in 20 ml of 0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5 and then incubated for 15 min at 37°C with Lumigen®-PPD (0.1 mg/ml; Boehringer Mannheim) in 1 ml of 0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5.

The membrane was sealed in Saran Wrap and incubated at room temperature for 1 hour prior to exposure to X-ray film (Fuji RX, Fisher Scientific, Tustin, CA) for 15 min. The developed film (Fig. 4) shows that the SEQ ID NO: 9 probe specifically detected *C.parvum* amplification products that are 307 base pair (Fig. 4, lanes 1 and 3) and 361 base pair (Fig. 4, lanes 1 and 4) in size. Moreover, the *C. parvum* specific probe did not hybridize with *G. lamblia* sequences from either multiplex reaction (Fig. 4, lanes 3 and 4)

Example 3

Differential Amplification and Detection of *Cryptosporidium* DNA from Oocysts

This example illustrates the use of *Cryptosporidium* genus specific primers to amplify DNA extracted from a variety of *Cryptosporidium* species. In addition, this example demonstrates the use of a *Cryptosporidium* species specific primer pair to selectively amplify *C. parvum* HSP70 DNA. Moreover, the example demonstrates differential detection of *C. parvum* amplification products using a species specific probe.

DNA was extracted from *C. parvum*, *C. muris* and *C. baileyi* oocysts by freezing in liquid nitrogen for 2 min, followed by thawing at 95°C for 5 min. Amplification reactions were conducted using primer pair SEQ ID NO: 2 and SEQ ID NO: 3 (see Fig. 5, lanes 1 to 3) or primer pair SEQ ID NO: 4 and SEQ ID NO: 5 (see Fig. 5, lanes 1 to 3) essentially as described in Example 1. The PCR products (15% of the amplification reaction) were detected by standard agarose gel electrophoresis and ethidium bromide staining, as shown in Fig. 5.

Primer pair SEQ ID NO: 2 and SEQ ID NO: 3 amplified nucleic acid sequences from *C. parvum* (Fig. 5, lane 1), *C. muris* (Fig. 5, lane 2), and *C. baileyi* (Fig. 5, lane 3). In contrast, primer pair SEQ ID NO: 4 and SEQ ID NO: 5 only amplified DNA target sequences from *C. parvum* (Fig. 5, lane 4).

A Southern blot of the gel shown in Fig. 5 was performed as described in Example 2. Moreover, hybridization with probe SEQ ID NO: 9, washing, and probe detection were also done essentially as described in Example 2. The *C. parvum* specific probe did not hybridize with the *C. muris* (Fig. 6, lane 2), and *C. baileyi* (Fig. 6, lane 3) amplification products, but did detect both *C. parvum* HSP70 amplification products (Fig. 6, lanes 1 and 4).

Example 4

Amplification Using Extracts from Infected Cell Cultures and Seeded Environmental Water Concentrates

This example illustrates methods for recovering and concentrating oocysts from water samples, In addition, the example illustrates an effective method for extracting template DNA from infected cells and oocysts for use in amplification reactions.

641 L of source water (0.65 NTU) was filtered through a 1 μm nominal porosity yarn-wound polypropylene filter. The filtration flow rate was restricted to about 4 liters/min. The filter fibers were then cut, teased and washed with a buffered detergent solution. Any oocysts or cysts that may have been eluted from the filter were concentrated by centrifugation and partially purified by flotation on a Percoll-sucrose solution with a specific gravity of 1.1. The final volume of the concentrated oocyst or cyst containing fraction was 1.3 ml. A 100 μl amount of this concentrate was seeded with 0.5 - 500 *C. parvum* oocysts (determined by serial dilution).

Total DNA was extracted from infected mammalian cell cultures and seeded

concentrates of source water samples (100 μl) by lysis in 50 m*M* Tris-HCl, 20 m*M* EDTA, pH 8, containing 2 mg/ proteinase K and 0.5% sarkosyl, followed by incubation at 37°C for 1 h. Then, 5 *M* NaCl was added to give a final concentration of 1 *M*, and CTAB was added to a concentration of 1%. Following incubation at 65°C for 30 min, the lysate was subjected to one freeze/thaw cycle and phenol/chloroform extraction. The DNA was precipitated by the addition of 0.6 vol of isopropanol, and the DNA precipitate was washed with 70% ethanol. After desiccation, the DNA pellet was resuspended in 100 μL of sterile distilled water.

The amplification reaction conditions using primer pair SEQ ID NO:4 and SEQ ID NO: 5 were generally the same as in Example 1. DNA was amplified by 40 cycles of denaturation at 94°C for 45 sec, annealing for 45 sec at 55°C and extension at 72°C for 1 min in reactions containing 1.5 mM $MgCl_2$. The seeded water concentrate amplification reactions also contained 10 μg/ml BSA.

PCR products (15% of the amplification reaction) were detected by standard agarose gel electrophoresis and ethidium bromide staining. DNA transfer, hybridization and detection were performed essentially as described in Example 2. The membrane was hybridized with the SEQ ID NO: 9 probe in 1 x SSC at 57°C for 18 h and washed in 1 x SSC at 54°C. The developed film (Fig. 7) shows that the primer and probe combination gave a strong detection signal whenever *C. parvum* specific sequences were present in the DNA extracts.

Although the present invention has been described in considerable detail with reference to certain preferred versions thereof, other versions are possible. For example, primers and probes can have additional nucleotide sequences that function as recognition sites for DNA-binding proteins. Therefore, the spirit and scope of the appended claims should not be limited to the description of the preferred versions described herein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: DeLeon, Ricardo
Rochelle, Paul
(ii) TITLE OF INVENTION: Cryptospo idium Dete tion Method
(iii) NUMBER OF SEQUENCES: 10
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Sheldon & Mak
(B) STREET: 225 S. Lake Aven e, 9th Floor
(C) CITY: Pasadena
(D) STATE: California
(E) ZIP: 91101
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 Mb storage
(B) COMPUTER: IBM compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: WordPerfect for Windows version 6.1
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: to be assigned
(B) FILING DATE: herewith
(C) CLASSIFICATION: to be ass gned
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Farah, David A.
(B) REGISTRATION NUMBER: 38,134
(C) REFERENCE/DOCKET NUMBER: 1364PCT
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (818) 796-4000
(B) TELEFAX: (818) 795-6321
(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3607 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double stranded
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCTCAT TCTATGGTGA AAGGTATATA TTATAAAATT TGTAATATTA TAATAAAATA 60

TTTTTTTCCT ATGAAATTTA ATTTTACAAG CATTAGTTTC ATAATATAAT CATATTGTTG 120

ATTAGTCTGT AAAAATATTA TTTGATTGAT GAGGAGATCC ATATTCACTT TATTTTATTA 180

GAATTTTTAT TAAATAAAAG TTGTATTATT TTTTTTTTAT TGTAATTATT AAAAATAATG 240

GCCTTTTTTT TTAATTAATA GAAATATTAT GTAGTGAATT ACAATCACAA CTTGATTTTA 300

CCATTTTATT ATATTTTTTT TAAAAATTAT TGTAATTATT TAATACTTTA CACACGTAAT 360

TTATTTTCTT CTATTGAATT AATTAAACTT TATTCCTATA ACATTCATAT ACAGTTGCAT 420

TGCAAATTTT GCATGCAATG CATGCATCAA TGTGGACAAA TTTTAATAAC GAAGCATGAA 480

CAACAACATG GCGGTTAGCT GCTAAAGTCA AATATTTTCA TTAATTATTA TTATAAGAAA 540

ACGAGGAGTT GATTTATTCG GAAAGTAAAG TGATAAAAGT AAATGGAAAA AAGGGAGAAA 600

ATGAGGAATA AGAGGGGGAA GAAATGAAAG AAAGAAAAAT ATAAGAGAAA GAATGGGAAG 660

AGTAGTAGTA GGAAGAAGGA AACAATGTAG TGGGAAATAT AACGCAATAA AAAAAAATGG 720

ACGCTACATG AGGGAAGTTT GAAATAGTTG ATAATTATAA ATTTTAATTT AAGTACAATT 780

TTTAATTCGA TTTCAATCTA CAAATACTAA TTAGAGAAAA TTATATGCAA TATTTTTTTT 840

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CCATGTTATA GAAAATTGAA GGGTTTAGGC GCCAAATCGA AGTTACTAC TTTGTATAAA    900

ATAATTTATA TATTAATTGC GCATTAAATA AAAATTAGGG GTTTGGCGG AATTCTGAG     960

ACGCAATAAT ATTTAAAATA ATAATAAATA ATCAAAATAT TAAGAAAAT GTAAATATAA   1020

TAAATGATGA AGAATAATGG TCAGTTGTTA AGTACGTATA AATGGCAAA TACTAATCAA   1080

AATGTAAAAA ATAAATAGTA TAATGTCAAA TTATGAATAA AAATAATAG ACACAGCTAT   1140

TTATGAGGGG AAAAGTCAAT GTGTCTCGTA AAGATTGAAA ATAAGTACA AGTTATTTTA   1200

TCTCACTAGA TAAATAGTTT ATTTCATTTT AACTGATTTA TAAATTCTT ATCGAAATTC   1260

AAAAAAACAA G ATG ACA TCA TCT GAA GGT CCA GCT ATT GGT ATT GAT TTA    1310
            Met Thr Ser Ser Glu Gly Pro Ala Ile Gly Ile Asp Leu
             1               5                  10

GGT ACC ACA TAC TCA TGT GTT GGT GTA TGG AGA AAC GAT ACT GTA GAT     1358
Gly Thr Thr Tyr Ser Cys Val Gly Val Trp Arg Asn Asp Thr Val Asp
     15                  20                  25

ATT GTT CCA AAC GAT CAA GGT AAC CGT ACA ACA CCA TCA TAT GTA GCA     1406
Ile Val Pro Asn Asp Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala
 30                  35                  40                  45

TTT ACA GAA ACT GAG CGT CTT ATT GGT GAT GCA GCA AAG AAC CAA GTT     1454
Phe Thr Glu Thr Glu Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val
                 50                  55                  60

GCT AGA AAC CCA GAA AAT ACA GTT TTT GAT GCT AAA CGT CTT ATT GGT     1502
Ala Arg Asn Pro Glu Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly
            65                  70                  75

AGA AAG TTT GAT GAT CAG GCA GTA CAA AGT GAT ATG ACT CAC TGG CCA     1550
Arg Lys Phe Asp Asp Gln Ala Val Gln Ser Asp His Thr His Trp Pro
        80                  85                  90

TTT AAA GTA GTT AGA GGT CCA AAG GAC AAG CCA ATC ATC AGT GTA AAC     1598
Phe Lys Val Val Arg Gly Pro Lys Asp Leu Pro Ile Ile Ser Val Asn
     95                  100                 105

TAC TTA GGT GAA AAG AAG GAA TTC CAT GCT GAA GAA ATT TCT GCT ATG     1646
Tyr Leu Gly Glu Lys Lys Glu Phe His Ala Glu Glu Ile Ser Ala Met
110                 115                 120                 125

GCT TTA CAA AAG ATG AAG GAG ATC TCT GAA GCA TAC TTG GGT CGC CAA     1694
Ala Leu Gln Lys Lys Lys Glu Ile Ser Glu Ala Tyr Leu Gly Arg Gln
                130                 135                 140

ATT AAG AAC GCT GTA GTT ACT GTT CCA GCT TAT TTC AAT GAC TCA CAG     1742
Ile Lys Asn Ala Val Val Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln
            145                 150                 155

CGT CAA GCA ACA AAG GAT GCA GGT GCA ATT GCT GGT TTG AAT GTA ATG     1790
Arg Gln Ala Thr Lys Asp Ala Gly Ala Ile Ala Gly Leu Asn Val Met
        160                 165                 170

AGA ATC ATT AAC GAG CCA ACT GCA GCT GCT ATT GCT TAT GGT CTT GAT     1838
Arg Ile Ile Asn Glu Pro Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp
    175                 180                 185

AAG AAA GGA ACT GGC GAG AGA AAT GTA TTG ATT TTC GAT TTA GGT GGT     1886
Lys Lys Gly Thr Gly Glu Arg Asn Val Leu Ile Phe Asp Leu Gly Gly
190                 195                 200                 205
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GGT ACT TTT GAT GTA TCA TTA TTA ACT ATT GAA GAT GGT ATT TTT GAA      1934
Gly Thr Phe Asp Val Ser Leu Leu Thr Ile Glu Asp Gly Ile Phe Glu
                210                 215                 220

GTT AAA GCT ACC GCT GGT GAT ACT CAC TTG GGT GGT GAA GAT TTT GAT      1982
Val Lys Ala Thr Ala Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp
            225                 230                 235

AAC AGA CTC GTA GAA TTC TGT GTA CAA GAT TTC AAG AGA AAG AAT AGA      2030
Asn Arg Leu Val Glu Phe Cys Val Gln Asp Phe Lys Arg Lys Asn Arg
        240                 245                 250

GGT ATG GAT TTA ACC TCA AAT GCT AGA GCT TTA AGA AGA CTC AGA ACT      2078
Gly Lys Asp Leu Thr Ser Asn Ala Arg Ala Leu Arg Arg Leu Arg Thr
    255                 260                 265

CAA TGC GAG CGT GCA AAG AGA ACT TTG TCA TCT TCT ACT CAA GCT ACA      2126
Gln Cys Glu Arg Ala Lys Arg Thr Leu Ser Ser Ser Thr Gln Ala Thr
270                 275                 280                 285

ATT GAG TTA GAT TCA CTC TAT GAA GGT ATT GAT TAT TCA GTT GCC ATC      2174
Ile Glu Leu Asp Ser Leu Tyr Glu Gly Ile Asp Tyr Ser Val Ala Ile
                290                 295                 200

AGT AGA GCT AGA TTC GAA GAA CTC TGC GCC GAT TAC TTC CGT GCA ACT      2222
Ser Arg Ala Arg Phe Glu Glu Leu Cys Ala Asp Tyr Phe Arg Ala Thr
            205                 210                 215

TTA GCT CCA GTT GAG AAA GTA CTC AAG GAT GCT GGT ATG GAC AAG AGA      2270
Leu Ala Pro Val Glu Lys Val Leu Lys Asp Ala Gly Met Asp Leu Arg
        220                 225                 230

TCT GTA CAT GAT GTT GTA TTG GTT GGT GGT TCT ACA CGT ATT CCA AAG      2318
Ser Val His Asp Val Val Leu Val Gly Gly Ser Thr Arg Ile Pro Lys
    235                 240                 245

GTT CAG GCC TTG ATT CAG GAA TTC TTT AAC GGT AAA GAG CCA TGC AAA      2366
Val Gln Ala Leu Ile Gln Glu Phe Phe Asn Gly Lys Glu Pro Cys Lys
250                 255                 260                 265

GCA ATC AAT CCA GAC GAA GCT GTT GCT TAT GGT GCT GCT GTA CAA GCT      2414
Ala Ile Asn Pro Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala
                270                 275                 280

GCT ATC TTA AAT GGT GAG CAA TCC TCT GCC GTA CAG GAT GTC TTA TTA      2462
Ala Ile Leu Asn Gly Glu Gln Ser Ser Ala Val Gln Asp Val Leu Leu
            285                 290                 295

TTG GAT GTT GCT CCA TTA TCA CTC GGT TTA GAA ACT GCT GGT GGT GTT      2510
Leu Asp Val Ala Pro Leu Ser Leu Gly Leu Glu Thr Ala Gly Gly Val
        300                 305                 310

ATG ACC AAG CTT ATT GAA CGT AAT ACA ACT ATC CCA GCA AAG AAG ACA      2558
Met Thr Lys Leu Ile Glu Arg Asn Thr Thr Ile Pro Ala Lys Lys Thr
    315                 320                 325

CAA GTC TTC ACT ACT TAT GCT GAT AAC CAG AGT GGT GTC TTG ATC CAA      2606
Gln Val Phe Thr Thr Tyr Ala Asp Asn Gln Ser Gly Val Leu Ile Gln
330                 335                 340                 345

GTT TAT GAG GGT GAG AGA GCC ATG ACT AAG GAT AAC CAT CTC CTC GGA      2654
Val Tyr Glu Gly Glu Arg Ala Met Thr Lys Asp Asn His Leu Leu Gly
                350                 355                 360

AAG TTC CAT CTT GAT GGT ATT CCA CCA GCA CCA AGA GGT GTA CCA CAA      2702
Lys Phe His Leu Asp Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln
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            365                 370                 375

ATT GAA GTC ACC TTT GAT ATT GAT GCT AAT GGT ATC TTG AAT GTG TCT      2750
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        380                 385                 390

GCT GTT GAT AAG AGT ACT GGT AAG AGC AGC AAG ATC ACT ATT ACT AAC      2798
Ala Val Asp Lys Ser Thr Gly Lys Ser Ser Lys Ile Thr Ile Thr Asn
    395                 400                 405

GAT AAG GGT AGA TTA TCA AAG GTA TCT GAT ATT GAA CGT ATG GTT AAT      2846
Asp Lys Gly Arg Leu Ser Lys Val Ser Asp Ile Glu Arg Met Val Asn
410                 415                 420                 425

GAT GCT GAG AAA TAC AAG GGT GAG GAT GAG CAG AAC AGA CTT AAG ATT      2894
Asp Ala Glu Lys Tyr Lys Gly Glu Asp Glu Gln Asn Arg Leu Lys Ile
                430                 435                 440

GAG GCT AAG AAC TCT TTG GAG AAC TAC CTC TAT AAC ATG AGG AAC ACC      2942
Glu Ala Lys Asn Ser Leu Glu Asn Tyr Leu Tyr Asn Lys Arg Asn Thr
            445                 450                 455

ATC CAA GAA CCA AAG GTT AAG GAA AAG CTT TCT CAA TCT GAA ATT GAT      2990
Ile Gln Glu Pro Lys Val Lys Glu Lys Leu Ser Gln Ser Glu Ile Asp
        460                 465                 470

GAG GCT GAG AAG AAG ATC AAG GAT GCT CTT GAC TGG CTC GAG CAC AAC      3038
Glu Ala Glu Lys Lys Ile Lys Asp Ala Leu Asp Trp Leu Glu His Asn
    475                 480                 485

CAA ACT GCT GAA AAG GAC GAG TTT GAA CAT CAA CAA AAG GAG ATT GAA      3086
Gln Thr Ala Glu Lys Asp Glu Phe Glu His Gln Gln Lys Glu Ile Glu
490                 495                 500                 505

ACT CAT ATG AAT CCA CTC ATG ATG AAG ATC TAC TCT GCT GAG GGT GGT      3134
Thr His Lys Asn Pro Leu His Met Lys Ile Tyr Ser Ala Glu Gly Gly
                510                 515                 520

ATG CCA GGT GGA ATG CCA GGT GGT ATG CCA GGC GGT ATG CCA GGT GGA      3182
Met Pro Gly Gly Met Pro Gly Gly Met Pro Gly Gly Met Pro Gly Gly
            525                 530                 535

ATG CCA GGT GGT ATG CCA GGT GGA ATG CCA GGC GGT ATG CCA GGT GGT      3230
Met Pro Gly Gly Met Pro Gly Gly Met Pro Gly Gly Met Pro Gly Gly
        540                 545                 550

ATG CCA GGT GGT ATG CCA GGT GGT ATG CCA GGA TCT AAT GGT CCA ACT      3278
Met Pro Gly Gly Met Pro Gly Gly Met Pro Gly Ser Lys Gly Pro Thr
    555                 560                 565

GTT GAA GAG GTC GAC TAATTATTTT AGTCACCAAA AAAACTCACT CAAAATGGAA      3333
Val Glu Glu Val Asp
570

AGTTAAGAAC TATTTACACA CTTTCAATTT CTAGTTATTT TTTACCAAAA TAAGAAGAAA    3393

AGCACACTCT ACCTTTAGGC TATATTTTCC ATTCTCTAGC CTAGACTCCC TTATATGCGA    3453

GTTGGCAATA TTTCACCAGA TTTACCGCCA TAAATTTGGC ATTTTTGGCT TATTGATAGT    3513

CATTACTATT ATCAATACGA GTTCTCGAAA AGAGAAAGCC CAGATATCTG GATAGTTTGG    3573

AACAAACTAT GTTCTCTAGT TTATTTGAGA ATTC                                3607
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: primer sequence
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:2:
CTGTTCCTTA TGGTGCTGCT G 21

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: primer sequence
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:3:
CCTCTTGGTG CTGGTGGAAT A 21

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: primer sequence
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:4:
AAATGGTGAG CAATCCTCTG 20

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: primer sequence
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:5:
CTTGCTGCTC TTACCAGTAC 20

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: primer sequence
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:6:
AGGGCTCCGG CATAACTTTC C 21

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: primer sequence
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:7:
GTATCTGTGA CCCGTCCGAG 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: probe sequence
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:8:
AAATGGTGAG CAATCCTCTG CCG 23

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: probe sequence
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:9:
CCATTATCAC TCGGTTTAGA 20

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: probe sequence
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:10:
CAGGCCTTGG CGTTCCCGAA G 21

WHAT IS CLAIMED IS:

1. A method of selectively detecting viable protozoan oocysts in a sample, which comprises:

a) recovering protozoan oocysts from a test sample;

b) elevating the temperature of the oocysts to a temperature sufficient to induce transcription of heat shock protein (HSP) RNA:

c) lysing the oocysts to liberate undegraded HSP RNA;

d) removing any DNA liberated from the lysed oocysts from the HSP RNA;

e) selecting a target HSP RNA sequence for a protozoa of interest and incubating the induced HSP RNA with a DNA polymerase having reverse transcriptase activity, four different deoxynucleotide triphosphates, and a first primer, wherein the first primer can hybridize with the target HSP RNA sequence and a cDNA/target RNA hybrid can be synthesized;

f) treating the cDNA/target RNA hybrid formed in step (e) to provide a single-stranded cDNA;

g) incubating the single-stranded cDNA formed in step (f) with a DNA polymerase, four different deoxynucleotide triphosphates, and a second primer, wherein the second primer can hybridize to the single-stranded cDNA and initiate synthesis of a double-stranded cDNA molecule; and

h) amplifying the double-stranded cDNA molecule of step (g) to form amplified target DNA; and

i) determining if amplified target DNA is present to test for viable oocysts in the test sample.

2. A method of selectively detecting infective protozoan oocysts in a sample, which comprises:

a) inoculating a cell culture with a sample suspected of having protozoan oocysts, wherein the cell culture is susceptible to infection by the protozoan oocysts;

b) incubating the cell culture under conditions sufficient for protozoan oocysts to infect the cell culture;

c) treating the cell culture to gain access to nucleic acids;

d) selecting a target heat shock protein (HSP) gene sequence, for a protozoa of interest, and incubating the nucleic acids with a DNA polymerase, four different deoxynucleotide

triphosphates, and a first primer, wherein the first primer can hybridize with the target gene sequence and initiate synthesis of a double-stranded polynucleotide sequence;

e) treating the double-stranded polynucleotide sequence formed in step (e) to provide a single-stranded polynucleotide sequence;

f) incubating the single-stranded polynucleotide sequence formed in step (e) with a DNA polymerase, four different deoxynucleotide triphosphates, and a second primer, wherein the second primer can hybridize to the single-stranded polynucleotide sequence and initiate synthesis of another double-stranded polynucleotide sequence;

g) amplifying the double-stranded polynucleotide sequence of step (g) to form amplified target DNA; and

h) determining if amplified target DNA is present to assay for infective oocysts in the test sample.

3. A method according to claim 2 wherein the nucleic acids are selected from a group consisting of DNA, RNA, and a combination of DNA and RNA.

4. A method according to claim 2 wherein the nucleic acids are RNA and the DNA polymerase has reverse transcriptase activity.

5. A method according to claim 2 wherein the cell culture comprises a cell number, the sample comprises a number of infective oocysts, and there is a ratio of the number of infective oocysts to the cell number (MOI) less than about 1.

6. A method according to claim 2 wherein step (b) further comprises adding an overlay to the cell culture to form infection foci.

7. A method according to claims 1 or 2 wherein the protozoa of interest is a *Cryptosporidium* species.

8. A method according to claim 7 wherein the protozoa of interest further comprises a *Giardia* species.

9. A method according to claim 7 wherein the *Cryptosporidium* species is selected from the group consisting of *C. parvum*, *C. muris*, *C. baileyi*, and *C. wrairi*.

10. A method according to claims 1 or 2 wherein the heat shock protein is HSP 70.

11. A method according to claim 10 wherein the protozoa of interest is *C. parvum*.

12. A method according to claim 11 wherein the target HSP 70 sequence is that portion of SEQ ID NO: 1 extending from about nucleotide 2370 to about 3607.

13. A method according to claim 11 wherein the target HSP 70 sequence is that

portion of SEQ ID NO: 1 extending from about nucleotide 2386 to about 2784.

14. A method according to claims 1 or 2 wherein the first primer is complementary and the second primer is homologous to portions of the HSP 70 gene sequence for *C. parvum*.

15. A method according to claim 14 wherein the first primer and second primer can hybridize with portions of the HSP 70 gene sequence for *C. parvum* that are conserved among *Cryptosporidium* species.

16. A method according to claim 15 wherein the first primer comprises all or a substantial part of SEQ ID NO: 3: 5'-CCT CTT GGT GCT GGT GGA ATA-3' and the second primer comprises all or a substantial part of SEQ ID NO: 2: 5'-CTG TTC CTT ATG GTG CTG CTG-3'.

17. A method according to claim 14 wherein the first primer and second primer can hybridize with portions of the HSP 70 gene sequence for C. *parvum* that are specific for C. *parvum*.

18. A method according to claim 17 wherein the first primer comprises all or a substantial part of SEQ ID NO: 5: 5'-CTT GCT GCT CTT ACC AGT AC-3' and the second primer comprises all or a substantial part of SEQ ID NO: 4: 5'-AAA TGG TCA GCA ATC CTC TG-3'.

19. A method according to claim 8 further comprising a third primer and a fourth primer, wherein the third primer is complementary and the fourth primer is homologous to portions of an HSP gene sequence of *Giardia*.

20. A method according to claim 18 wherein the third primer comprises all or a substantial part of SEQ ID NO: 7: 5'-GTA TCT GTG ACC CGT CCG AG-3' and the fourth primer comprises all or a substantial part of SEQ ID NO: 6: 5'-AGG GCT CCG GCA TAA CTT TCC-3'.

21. A method according to claim 1 or 2 wherein the amplification is accomplished by the polymerase chain reaction.

22. A method according to claim 1 or 2 wherein the presence of amplified target DNA is determined by subjecting the amplified target DNA to hybridization conditions with a probe complementary to a heat shock protein gene sequence.

23. A method according to claim 22 wherein the probe is homologous to a HSP 70 gene sequence.

24. A method according to claim 23 wherein the HSP 70 gene sequence is the HSP 70

gene sequence for *C. parvum*.

25. A method according to claim 24 wherein the probe is homologous to portions of the HSP 70 gene sequence for *C. parvum* specific for *Cryptosporidium parvum*.

26. A method according to claim 25 wherein the probe comprises all or a substantial part of SEQ ID NO: 8: 5'-AAA TGG TGA GCA ATC CTC TGC CG-3' or its complement.

27. A method according to claim 25 wherein the probe comprises all or a substantial part of SEQ ID NO: 9: 5'-CCA TTA TCA CTC GGT TTA GA-3'or its complement.

28. A method according to claim 22 wherein the probe comprises a first probe and a second probe, the first probe is homologous to portions of the HSP70 gene sequence for *C. parvum* and the second probe is homologous to portions of an HSP gene sequence for *G. lamblia*.

29. A method according to claim 28 wherein the second probe comprises all or a substantial part of SEQ ID NO: 10: 5'-CAG GCC TTG GCG TTC CCG AAG-3' or its complement.

30. A kit for use in a process for detecting *Cryptosporidia*, the kit comprising a first primer and a second primer for amplification of a target sequence in an HSP 70 gene and a probe sequence for detection of an amplified target sequence in the HSP 70 gene.

31. A kit according to claim 30 wherein the first primer comprises all or a substantial part of SEQ ID NO: 3: 5'-CCT CTT GGT GCT GGT GGA ATA-3' and the second primer comprises all or a substantial part of SEQ ID NO: 2: 5'-CTG TTC CTT ATG GTG CTG CTG-3'.

32. A kit according to claim 31 wherein the probe comprises all or a substantial part of SEQ ID NO: 8: 5'-AAA TGG TGA GCA ATC CTC TGC CG-3' or its complement.

33. A kit according to claim 30 wherein the first primer comprises all or a substantial part of SEQ ID NO: 5: 5'-CTT GCT GCT CTT ACC AGT AC-3' and the second primer comprises all or a substantial part of SEQ ID NO: 4: 5'-AAA TGG TCA GCA ATC CTC TG-3'.

34. A kit according to claim 33 wherein the probe comprises all or a substantial part of SEQ ID NO: 9: 5'-CCA TTA TCA CTC GGT TTA GA-3'or its complement.

35. A kit for use in a process for detecting *Cryptosporidium* and *Giardia*, the kit comprising a first primer and a second primer for amplification of a first target sequence in a *Cryptosporidium* HSP 70 gene, a first probe sequence for detection of an amplified first target

sequence in the *Cryptosporidium* HSP 70 gene, a third primer and a fourth primer for amplification of a second target sequence in a Giardia HSP gene, a second probe sequence for detection of an amplified second target sequence in the *Giardia* HSP gene.

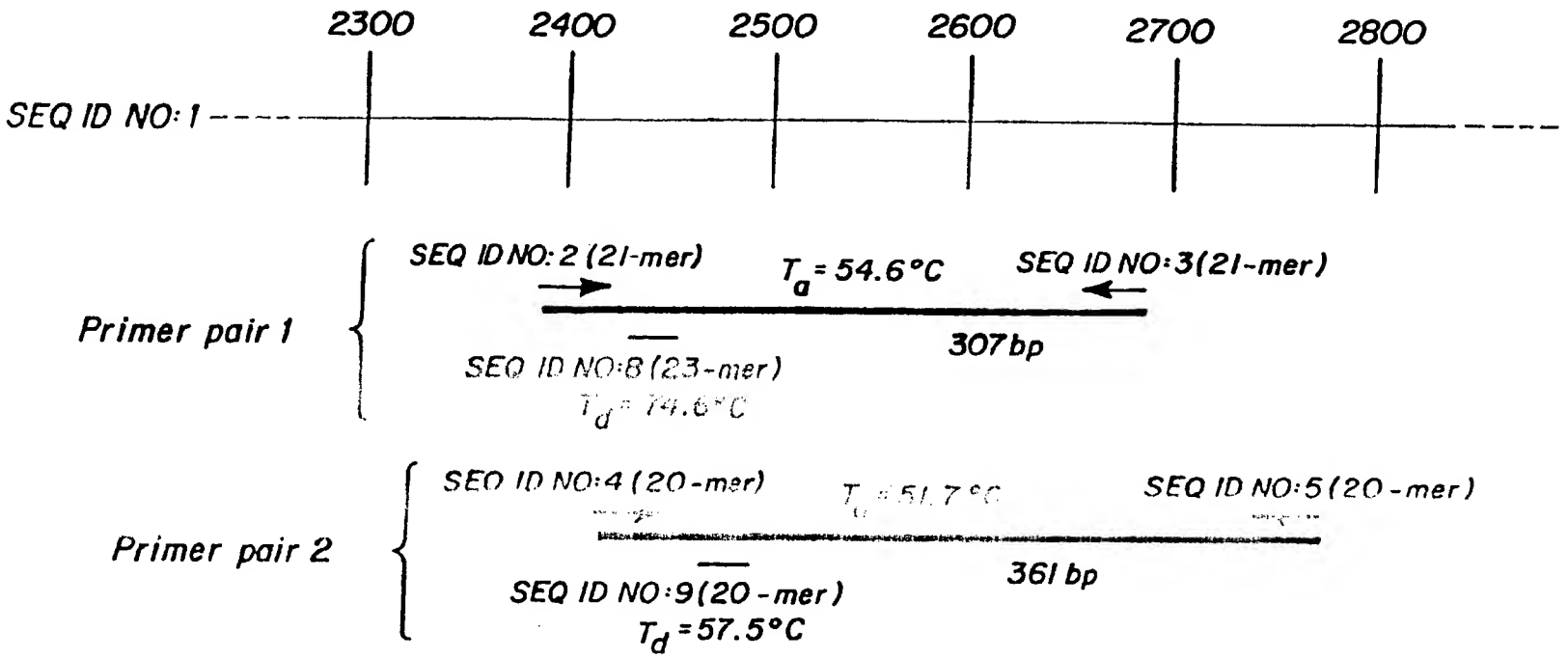
36. A kit according to claim 35 wherein the first primer comprises all or a substantial part of SEQ ID NO: 3: 5'-CCT CTT CGT GCT TGT GGA ACA-3', the second primer comprises all or a substantial part of SEQ ID NO: 4: 5'-CTG TTC CTT ATG GTG CTG CTG-3', the third primer comprises all or a substantial part of SEQ ID NO: 7: 5'-GTA TCT GTG ACC CGT CCG AG-3' and the fourth primer comprises all or a substantial part of SEQ ID NO: 6: 5'-AGG GCT CCG GCA TAA CTT TCC-3'.

37. A kit according to claim 36 wherein the first probe comprises all or a substantial part of SEQ ID NO: 8: 5'-AAA TGG TGA GCA ATC CTC TCC CG-3' or its complement and the second probe comprises all or a substantial part of SEQ ID NO: 10: 5'-CAG GCC TTG GCG TTC CCG AAG-3' or its complement.

38. A kit according to claim 35 wherein the first primer comprises all or a substantial part of SEQ ID NO: 5: 5'-CTT GCT GCT CTT ACC AGT AC-3', the second primer comprises all or a substantial part of SEQ ID NO: 4: 5'-AAA TGG TCA GCA ATC CTC TG-3', the third primer comprises all or a substantial part of SEQ ID NO: 7: 5'-GTA TCT GTG ACC CGT CCG AG-3' and the fourth primer comprises all or a substantial part of SEQ ID NO: 6: 5'-AGG GCT CCG GCA TAA CTT TCC-3'.

39. A kit according to claim 36 wherein the first probe comprises all or a substantial part of SEQ ID NO: 9: 5'-CCA TTA TCA CTC CGT TTA GA-3' or its complement and the second probe comprises all or a substantial part of SEQ ID NO: 10: 5'-CAG GCC TTG GCG TTC CCG AAG-3' or its complement.

FIG. 1



→ PCR primer

— internal probe

━━ PCR product

T_a calculated optimum PCR annealing temperature

T_d dissociation temperature

(21-mer) oligonucleotide length

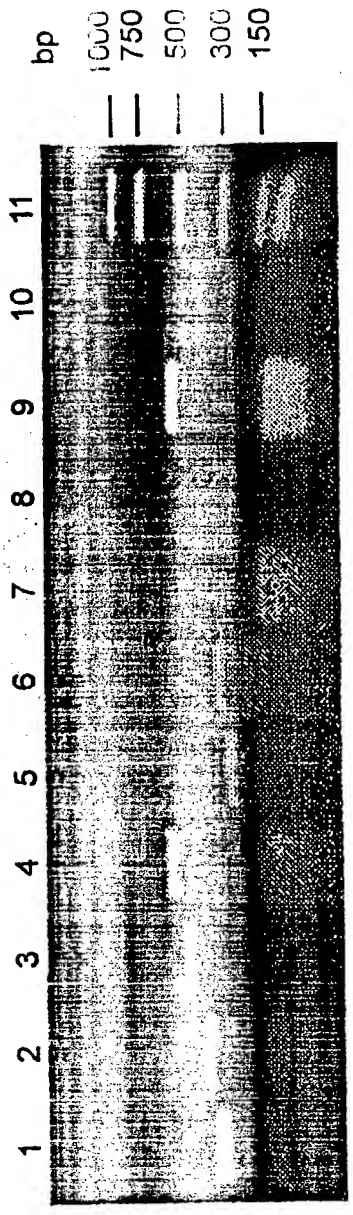


FIG. 2

FIG. 3

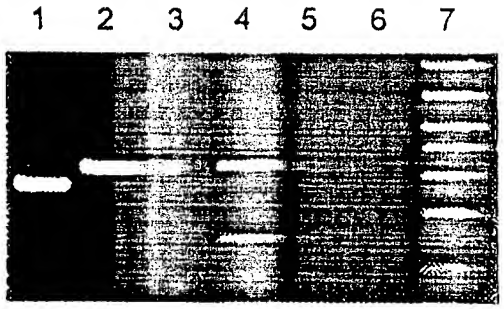


FIG. 4

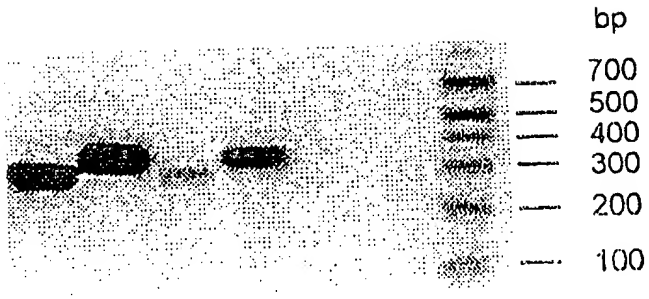


FIG. 5

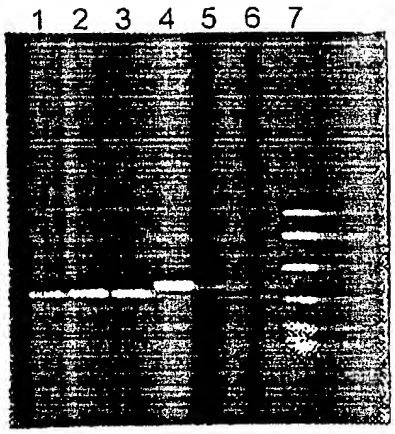


FIG. 6

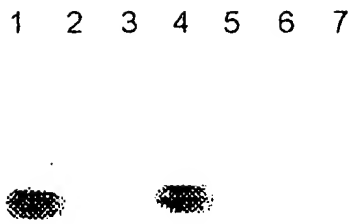
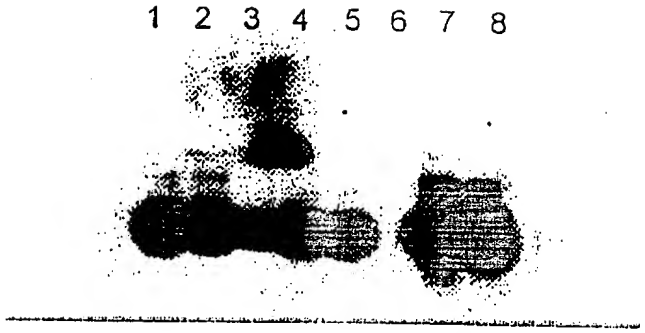


FIG. 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/07972

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34; C07H 21/04
US CL :435/6, 91.2; 536/24.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/24.33; 435/32, 34, 39, 91

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,908,308 A (VAN DER PLOEG et al) 13 March 1990, see entire document, especially the abstract and columns 1-6).	1-39
Y	US 5,298,392 A (ATLAS et al) 29 March 1994, see entire document.	1-39
Y	US 4,983,511 A (GEIGER et al) 08 January 1991, see entire document, especially columns 1-2).	1-39
Y	ABBASZADEGN et al. Detection of Viable Giardia cysts in water samples using polymerase chain reaction. In Proceedings American Water Works Association, Water Quality Tecnology Conference. Toronto, 1993, pages 529-541, see entire document.	1-39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means		
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Date of the actual completion of the international search	Date of mailing of the international search report
06 JULY 1997	14 AUG 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer IREM YUCEL

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/07972

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AGGARWAL et al. A Heat Shock Protein gene in Giardia lamblia Unrelated to HSP70. Nucleic Acids Research. 1990, Vol. 18, No. 11, page 3409.	1-6, 8, 9, 19-23, 28-30, 35-39
Y	KHRAMTSOV et al. Cloning and Analysis of a Cryptosporidium parvum Gene Encoding a Protein with Homology to Cytoplasmic Form Hsp70. Journal of Eukaryotic Microbiology. 1995, Vol. 42, No. 4, pages 416-422, see especially Figures 2 and 3.	1-7, 9-18, 21-39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/07972

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, MEDLINE, WPID, BIOSIS, SCISEARCH

search terms: protozoa? detect? viability, infectious, heat shock, protein, hsp 70, PCR, assay, cryptosporidium, giardia